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Note

Determination of 3-deazaguanine in mice plasma by high-performance liquid chromatography

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3-Deazaguanine (3-DG), 6-amino-1,5-dihydro-4H-imidazo[4,5-C]pyridin-4-one is a synthetic analogue of guanine in which the nitrogen in the 3-position has been replaced with a carbon and hydrogen atom [1]. 3-DG has been shown to be a potential antitumor and antiviral agent [2]. The growth inhibitory activity of 3-DG in bacterial as well as in mammalian systems, has been shown to be the result of its conversion to 3-DG-containing nucleotides [3,4]. 3-DG inhibited several cell types including L1210 [5,6], HeLa [7], Ehrlich ascites [4], EMT-6 [8], primary Chinese hamster embryo cells [9], hamster ovary cells [10] and KB cells [4]. 3-DG has been presumably reported to inhibit the two enzymes involved in the purine biosynthetic pathway namely, inosine monophosphate dehydrogenase (IMPDH) and hypoxanthine—guanine phosphoribosyl transferase (HGPRT) which are probably the sites of cellular action.

To understand the fate of 3-DG in the biological system and also to study the mechanism of action of the drug in detail, we developed a sensitive high-performance liquid chromatographic (HPLC) method of determination of 3-DG and the efficacy of the method is analyzed in plasma and urine of mice and the results of the observation are discussed in this communication.

EXPERIMENTAL

Apparatus

Separation was achieved on a Model 6000 A solvent delivery system, Model U6K universal injector (Waters Assoc., Milford, MA, U.S.A.) and Model SF-720 spectroflow monitor (Schoeffel Instruments, Westwood, NJ, U.S.A.). Peak areas, retention time and concentrations based on standards were calculated with a Model 720 system controller and a Model 730 data module (Waters Assoc.).

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Column

The column used for reversed-phase HPLC was 300 × 4 mm μBondapak C_{18} (Waters Assoc.). The column was prepacked with 10-μm (average diameter) porous silica particles to which octadecyl groups were covalently bonded through a Si—O—Si bond.

Reagents

Ammonium formate (Sigma, St. Louis, MO, U.S.A.), trichloroacetic acid (Baker, Phillipsburg, NJ, U.S.A.), methanol (HPLC grade, Fisher, Fairlawn, NJ, U.S.A.) and glass-double-distilled water were used in preparing buffers and all other aqueous solutions. All solutions used in the HPLC system were filtered through a membrane filter (average pore size 0.2 μm; Millipore, Bedford, MA, U.S.A.) and degassed under vacuum immediately prior to use in the HPLC system.

Drug

3-Deazaguanine was obtained from Warner-Lambert (Ann Arbor, MI, U.S.A.). A stock solution of 10 mg/ml of the drug was prepared in 0.05 *M* hydrochloric acid and further dilutions were made with glass-distilled water and used as standards.

Internal standard

Fluorouridine (FUR) was used as the internal standard for 3-DG. A stock solution of 10 m*M* FUR was prepared in glass-distilled water and stored at −20°C and further dilutions were made immediately prior to use.

Buffer

A solution of 5 m*M* ammonium formate was freshly prepared in glass-double-distilled water and filtered through a membrane filter and degassed under vacuum immediately prior to use in the HPLC system.

Sample collection and storage

Female B6DZF_1 mice (20—23 g) were used in all the experiments. The animals were housed under natural lighting and fed a standard laboratory chow (Wayne Lab. Animal Diets, Chicago, IL, U.S.A.) ad libitum. Each mouse was administered intraperitoneally with 0.2 ml of 3-DG in normal saline (5 mg/ml). About 0.5 ml of blood was collected at different time intervals (0, 2, 5, 15, 30, 60, 90, 120, 240, 360, 720, 1440 min) from retroorbital sinus of mice using heparinized microhematocrit capillary tubes and immediately centrifuged at 12,000 *g* for 2 min. Plasma was separated and stored in ice until the collection was completed and then kept frozen at −20° C.

Reversed-phase HPLC determination of 3-DG

A 25-μl aliquot of plasma was placed in Eppendorff microcentrifuge tubes and 75 μl of 6% trichloroacetic acid were added to deproteinize plasma and vortexed with 100 μl of water. The solutions were centrifuged for 5 min and an aliquot of 20 μl was injected and chromatographed. The drug was monitored by its absorbance at 254 nm. The areas under the peaks were integrated with

the data module and system controller and the amount of drug in the plasma was calculated as follows: The amount of drug per ml of sample:

$$\left[\frac{\text{Area}_{\text{test}}}{\text{Area}_{\text{standard}}}\right]_{\text{sample}} \times \text{amount of standard} \times \frac{\text{ml sample}}{\text{volume of standard}}$$

Animal experiment

3-DG was injected into mice, three in each group. Plasma levels of drug were analyzed in a total number of eighteen mice, which were divided into six groups of three mice in each group. Blood was drawn twice from each mouse, i.e. a total number of 36 times was drawn from eighteen mice at various time intervals as described earlier.

HPLC conditions

Initially, the column was in 100% methanol phase and the column was washed with glass-distilled water for 30 min and regenerated for drug analysis by pumping 5 m*M* ammonium formate for a further 30 min. Flow-rate was maintained at 1 ml/min, the temperature was 24°C at 0.01 a.u.f.s.

RESULTS AND DISCUSSION

A chromatogram demonstrating the resolution of 3-DG is shown in Fig. 1. It can be seen from Fig. 1 that 3-DG has a retention time of 11 min at a flow-rate of 1 ml/min and it is sufficiently resolved to allow accurate quantitation. Identification of the peaks found in the chromatograms of plasma or urine were observed by matching the retention times of peaks from plasma or urine with those of standard compounds. Further confirmation was achieved by addition of a known quantity of each standard to a duplicate sample of plasma or urine containing drug, followed by chromatography of both samples. It is evident that the characteristics of the identified peaks remained unchanged, although they were appropriately taller with the added standard, and no new peaks emerged.

Separation of 3-DG and FUR is shown in Fig. 2. FUR has the retention time of 16.5 min at the flow-rate of 1 ml/min. There is no non-specific peak corresponding to FUR in control plasma or urine.

Quantitation of 3-DG at different concentrations ranging from 100 ng/ml to 1 mg/ml showed that the linearity of the drug was observed in the concentrations ranging from 1.0 to 100 μg/ml. The peak areas corresponding to 3-DG concentrations of 1, 5, 10, 50 and 100 μg/ml were $20.63 \cdot 10^4$, $109 \cdot 10^4$, $219 \cdot 10^4$, $1024 \cdot 10^4$ and $2072 \cdot 10^4$, respectively. The efficiency of the HPLC separation allows a low detection of 100 ng/ml or 2 ng per injection.

The imprecision of the determination is a correlation of the relative standard deviation of the yield and this amounts to less than 1% for the blood plasma determination of 3-DG (Table I). The yield of procedure was determined by adding 40, 20 and 10 μg of 3-DG to each milliliter of three normal plasma samples and then processing the samples as described in the Experimental

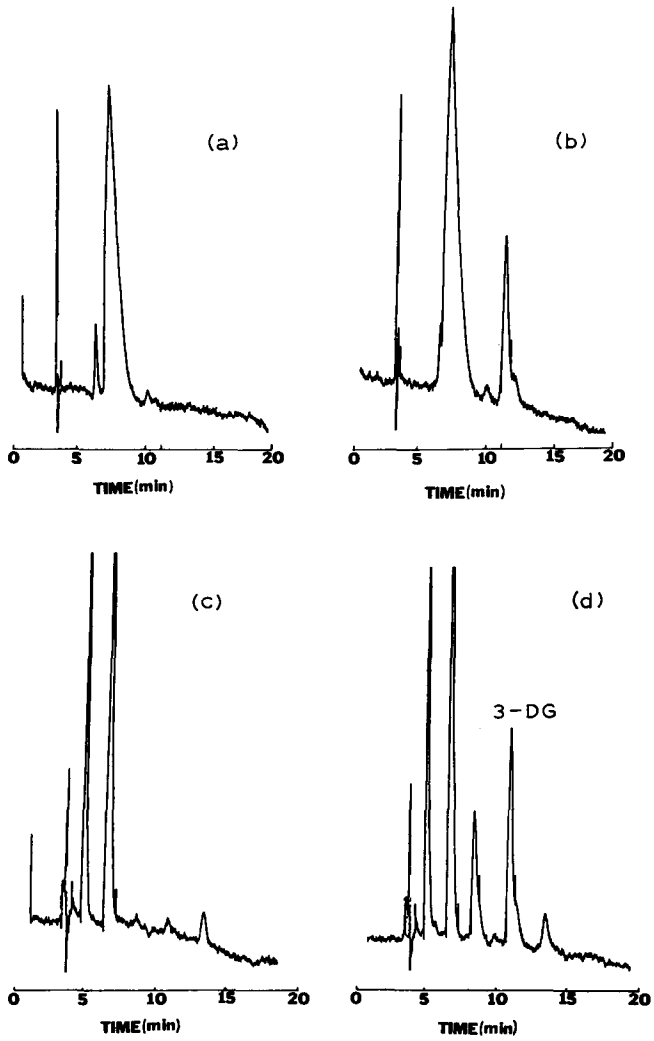

Fig. 1. Chromatograms obtained from control plasma (a); plasma containing drug (b); control urine (c); and urine containing drug (d). Plasma and urine were spiked with 3-DG to obtain a concentration of 2.5 μg/ml and 5.0 μg/ml, respectively and the deproteinized supernatants were chromatographed. Column: μBondapak C_{18} (300 × 4 mm); buffer: 5 m*M* ammonium formate (native pH); flow-rate: 1 ml/min; detector: 254 nm; 0.01 a.u.f.s.; temperature: 24°C.

section. The mean overall yield was found to be 91.7 ± 3.7%. The correction factor for the yield is therefore 1.09 in plasma.

When plasma was spiked with 40, 20 and 10 μg/ml 3-DG and aliquots were processed as described in Experimental and quantitated at daily intervals for a week, it was shown that the drug is stable in plasma under the experimental conditions. When the above experiment was repeated by spiking urine with 3-DG and immediately filtering through a membrane filter and chromatographing an aliquot, there was about 40—50% loss of parent drug in urine. This loss could be accounted for by the presence of a new peak which has a retention time of about 8 min (Fig. 1c and d).

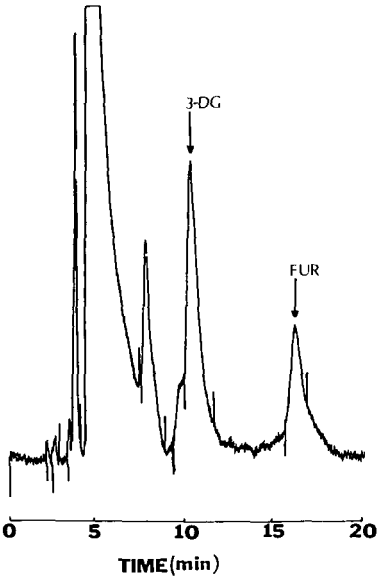

Fig. 2. Chromatogram representing the separation of 3-DG and the internal standard FUR in plasma. Conditions are the same as in Fig. 1.

TABLE I

PRECISION OF THE HPLC ASSAY OF 3-DEAZAGUANINE

$n = 3$.

Compound	Amount injected (μg/ml)	Peak area*			Mean ± S.D. (%)
3-Deazaguanine	10	219	219	218	218.66 ± 0.21
	5	110	111	110	110.33 ± 0.21
	1	21.4	21.5	21.5	21.466 ± 0.21

*Actual peak area = peak area $\times 10^4$.

We made an attempt to quantitate 3-DG in 24-h urine specimens of mice after administration of the drug but we could not detect the presence of the drug in the first and second 24-h urine specimens.

When an aliquot of 20 μl filtrate of control plasma was chromatographed, there was no non-specific peak corresponding to 3-DG. Retention times and peak areas in the chromatograms of authentic drug and plasma containing drug were found to be the same at isocratic conditions and so no attempt was made to purify plasma further for drug assay. Analysis of drug in plasma showed the presence of drug (5.0 ± 0.52 μg/ml) within 2 min after administration, reaching a maximum at 5 min (6.1 ± 0.68 μg/ml) and steadily decreasing thereafter. Drug could be quantitated in up to 2-h specimens (0.37 ± 0.02 μg/ml) and it disappeared completely from blood by 3 h (Fig. 3).

FUR was selected to serve as internal standard in the determination of 3-DG since the two compounds have the same absorption maxima, extraction and precision properties. It is also a readily available compound and its retention time under the experimental conditions is quite convenient to use it as an internal standard for 3-DG.

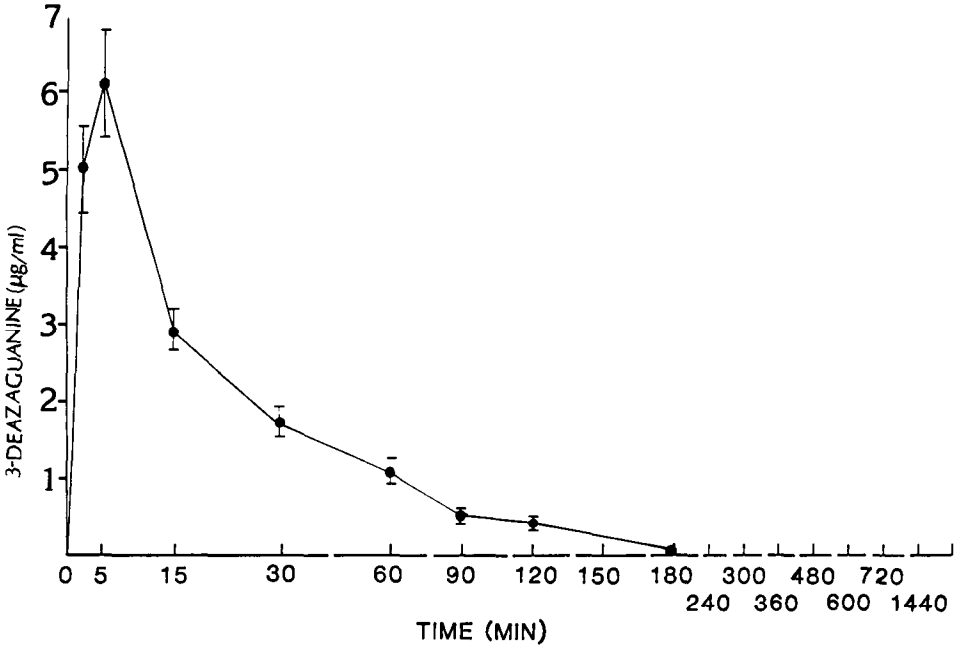

Fig. 3. Analysis of 3-DG in mice plasma. A 1-mg amount of 3-DG was injected into each mouse and a minimum number of three blood withdrawals of mice were quantitated at every time point.

The reversed-phase HPLC method for the separation and quantitation of 3-DG from plasma with UV absorption detection is a rapid, efficient, selective, sensitive and quantitative method. There is no method available in the literature for the estimation of the drug. 3-DG is soon to enter Phase I study. The elucidation of the method will throw some light on the pharmacology of the drug.

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